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Yq Microdeletions—Azoospermia Factor Candidate Genes and Spermatogenic Arrest

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In the last few years considerable progress has been made in the study of sperm physiology and the biology of gamete interaction, furthering our understanding of the pathophysiology of male infertility. With the advent of assisted reproductive technology and intracytoplasmic sperm injection, study of the various factors leading to spermatogenic impairment has become a major focus of scientific research. Understanding the genetic factors that lead to infertility has taken on a certain urgency, as we have learned not only of the transmission to male offspring of spermatogenic impairment, but that these offspring may also be born with a secondary, larger deletion with worsening of phenotype and genital ambiguity.

Ten to twenty-five percent of couples encounter difficulty procreating. Microdeletions of the long arm of the Y chromosome are associated with spermatogenic failure and have been used to define three regions on Yq (AZFa, AZFb, and AZFc) that are critical for spermatogenesis. This study was conceived in order to identify the frequency of submicroscopic interstitial deletions in azoospermia factor loci in infertile Indian males. One hundred and seventy five males with nonobstructive oligozoospermia and azoospermia were included in this study. Semen analysis was done in each case to determine the spermatogenic status—normospermic, oligozoospermic (< 20 million sperm/mL), or azoospermic (no sperm in the semen). Detailed medical, clinical, reproductive, and family histories were taken of each patient. Thirty G-banded metaphases were analyzed in each case and polymerase chain reaction microdeletion analysis was done in 133 cytogenetically normal cases. For this genomic, DNA was extracted using peripheral blood. The sequence tagged site primers tested in each case were sY84, sY86 (AZFa); sY113, sY116, sY127, sY134 (AZFb); sY254, sY255 (AZFc). Polymerase chain reaction amplifications found to be negative were repeated at least three times to confirm the deletion of a given marker. The polymerase chain reaction products were analyzed on a 1.8% agarose gel. Eight of the 133 cases showed deletion of at least one of the sequence tagged site markers. Review of the literature has shown that the overall frequency of microdeletions varies from 1% to 55%. In the present study the frequency of microdeletion was 6.01%. Deletions were detected in cases with known and unknown etiology with bilateral severe testiculopathy.

Key Words: Azoospermia factor, oligozoospermia, azoospermia, infertility.

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Azoospermia factor (AZF) microdeletions are a prevalent cause of male factor infertility. Male infertility is a major health problem today and 40% to 50% men in the reproductive age group have qualitative or quantitative abnormalities in sperm production. In more than 60% of cases the origin of reduced testicular function is unknown.[1,2] Several studies have reported a marked decline in male reproductive health and an increase in the population of subfertile males. Both genetic and environmental factors are believed to be responsible for this decline. Another factor that is reported to cause DNA damage in the sperm and Y chromosome is oxidative stress, in which there is a decline in the total antioxidant capacity in the semen of infertile males and increased levels of reactive oxygen species.

Germ cell development is under the control of a large number of genes on autosomes and on the Y chromosome. The long arm of the Y chromosome contains genes and gene families involved in spermatogenesis and is critical for germ cell development and differentiation. It has been known since 1970 that deletion of the long arm of the Y chromosome is associated with spermatogenic failure and leads to partial or complete spermatogenic arrest. It is only in the last few years that the loci involved in production and differentiation of sperm have been identified using molecular methods. The Y chromosome has been divided into seven deletion intervals. Each of these intervals is further subdivided into subintervals (A, B, C, etc).[3] In 1992, Vollrath and colleagues[4] constructed a 43-interval deletion map of a human Y chromosome that contained an ordered array of sequence tagged sites (STS) that spanned the entire length of the Y chromosome. The genes critical for spermatogenesis are located on the long arm of the Y chromosome in deletion interval 5 and 6 band 11.23. This region is referred to as the AZF, as the most severe phenotype associated with its deletion is azoospermia. The AZF region has three nonoverlapping loci—AZFa, AZFb, and AZFc—deletions of which are associated with spermatogenic failure.[5] The AZFa locus is located on proximal Yq11 (Yq11.21), while AZFb and AZFc are located on distal Yq11 (Yq11.23). Deletion of these loci results in spermatogenic arrest and is associated with azoospermia, oligozoospermia, and also with a varied testis histological profile ranging from Sertoli cell only (SCO), to hypospermatogenesis, to maturation arrest.[5] These AZF genes code for RNA binding proteins and may be involved in the regulation of gene expression, RNA metabolism, packaging and transport to cytoplasm, and RNA splicing. In order to gain information about AZF deletions in infertile Indian males and their relation to testicular phenotype, we decided to analyze the DNA of cytogenetically normal men with severe testiculopathy.

MATERIALS AND METHODS

The study included 175 infertile men and 50 age-matched fertile controls. The diagnosis of azoospermia and oligozoospermia was made on the basis of semen analyses according to guidelines established by the World Health Organization.[6] Semen was analyzed for motility, morphology, count, pH, and viability of sperm. Only cases with nonobstructive oligozoospermia and azoospermia were included in the study. Each patient was carefully examined to rule out other causes of infertility, and detailed medical, clinical, reproductive, and family histories were taken on a predesigned performa. Peripheral blood cultures were set up for chromosomal analysis and five well spread G-banded metaphases were karyotyped using the standard protocols. All cases with azoospermia and oligozoospermia from obstructive causes and cases with constitutional cytogenetic abnormality were excluded for molecular analysis using polymerase chain reaction (PCR) analysis. The values of follicular stimulating hormone and testosterone levels were recorded for all patients. Whenever possible, testicular cytopathological details following testicular fine needle aspiration cytology (FNAC) were collected, as testicular biopsy was ethically not possible in these cases. Patient consent was received in each case and ethical clearance was obtained prior to the study from the ethical clearance committee of the All India Institute of Medical Sciences.

PCR Analysis

In 133 infertile men with a normal 46,XY karyotype, molecular analysis for AZF loci was done using PCR. Each patient was examined for eight AZF loci that mapped to interval 5 and 6 of the Y chromosome. The SRY-sex-determining region on the short arm of Y chromosome (sY14) was used as an internal control. The following STS primers were used: sY84, sY86 (AZFa); sY113, sY116, sY127, sY134 (AZFb); and sY254, sY255 (AZFc). This primer set was suggested by Simoni et al.[7] and is recommended by the European Academy of Andrology. It enables the detection of over 90% of deletions in the AZF locus and allows for minimal standardization and comparison of the data on AZF deletions from different laboratories in different countries. Samples were subjected to PCR amplification using 35 cycles at 95°C for 1 min, 56°C for 30 sec, and 72°C for 30 sec. Initial denaturation was done for 5 min at 95°C and final extension time of 7 min at 72°C was given. An STS was considered absent only after at least three amplification failures in the presence of successful amplification of control (SRY-sY14).

PCR Controls

Fertile male and female samples were used as positive and negative controls and water was used as blank to check for false positive and false negative results.

The PCR products were analyzed on a 1.8% agarose gel containing ethidium bromide (0.5 μg/mL).

RESULTS

Of the 175 infertile men, 149 were azoospermic and 26 were oligozoospermic. Thirty-nine of the 175 men had the following chromosomal abnormalities and were not analyzed further at the molecular level: Klinefelter syndrome 47,XXY (n = 12); mosaic Klinefelter (n = 14); Klinefelter variants (n = 8); 46,XX (SRY negative) male (n = 1); 46,XX(80%)/47,XYY(20%) (n = 1); 46,XX(90%)/46,XY(10%) (n = 1); 46,XYdel1q, 46,XYq– (n = 1); 46,XY del 1qh+ (n = 1).

Polymerase chain reaction microdeletion analysis was done in 133 infertile patients and 50 fertile controls.

Polymerase chain reaction amplification produced a band of expected size for all the nine loci investigated in 125 infertile patients and 50 fertile controls. Eight patients (6.01%)—4 with idiopathic infertility and 4 with nonidiopathic infertility—showed a deletion of one or more STS, namely sY84, sY86, sY113, sY116, sY127, sY134 (patients 1 and 2); sY84, sY86, sY113, sY116, sY127 (patient 3); sY113, sY116, sY127, sY134 (patient 4); and sY254, sY255 (patients 5, 6, 7, and 8). All deletions were interstitial. Polymerase chain reaction amplification performed on the fathers of 2 of these 8 patients (patients 4 and 5) did not show microdeletions and thus the deletions in these 2 patients were de novo. The fathers of the other 6 patients with microdeletions (patients 1, 2, 3, 6, 7, 8) were not available.

In summary, patients 1 and 2 had complete deletion of AZFa and AZFb loci, patient 3 had complete AZFa deletion and partial deletion of AZFb, and patient 4 had AZFb deletion alone. In cases 5, 6, 7, and 8 there were deletions of AZFc loci. Seven of these 8 patients (patients 1, 2, 3, 4, 5, 7, 8) were azoospermic and patient 6 was oligoasthenoteratozoospermic with a total mean sperm count of 1.5 million, which declined to 0.2 million after one year. He had a high percentage of morphologically abnormal sperm with very few (< 12%) sperm with linear progressive motility (Fig. 1). Patients 1, 2, and 3 with AZFa and AZFb microdeletions had complete absence of germ cells in the seminiferous tubules and presence of Sertoli cells only (a picture characteristic of SCO syndrome). Patients 6 and 8 with AZFc deletions showed hypospermatogenesis, and patient 7 with AZFc deletion had maturation arrest at the secondary spermatocyte stage. Patients 4 and 5 with AZFb and AZFc deletions, respectively, were cryptorchid, and thus testicular FNAC was not possible. Patient 3 had left-sided varicocele and deletions of AZFa and partial AZFb, and patient 8 had AZFc deletion. The mean value in these 8 patients for follicular stimulating hormone was 30.4 mIU/mL (normal 1.2–5.0 mIU/mL). Clinical details of these eight patients with microdeletions are shown in Table 1.

DISCUSSION

Spermatogenesis is regulated by a number of genes on the Y chromosome and autosomes. Y-chromosome deletions are emerging as a prevalent cause of male factor infertility. The frequency of Y-chromosome deletion increases with the severity of spermatogenic defect.[8] About 15% of azoospermic and 5% to 10% of oligozoospermic men show Y-chromosome deletions. However, these Y-chromosome microdeletions cannot be predicted cytogenetically, on the basis of clinical findings, or by semen analysis. Thus, PCR-based AZF screening for Yq microdeletions is necessary. In the past, the diagnosis of a genetic etiology had little clinical significance. But today, with the advent of assisted reproductive technology and our knowledge of the vertical iatrogenic transmission of these genetic anomalies to the offspring, diagnosing the presence of these deletions has become very important. Diagnosis not only aids in determining the prognosis in these infertile cases but provides the information necessary to counsel these couples effectively, particularly with regard to the birth of infertile male offspring who may have the same or secondary, larger deletions with more severe testicular phenotype.

In this study, 4 infertile azoospermic and oligozoospermic men with idiopathic infertility, 2 men with cryptorchidism, and 2 men with left-sided varicocele harbored microdeletions spanning the AZF region.

Efforts have also been made to determine the incidence of Yq microdeletions and to correlate the size and position of the Yq deletion with the infertile phenotype. Recent studies have shown a marked variation in the deletion frequency. This is due to selection of different patient groups and use of different marker sets.

Vogt et al.[5] correlated the position of the AZF deletion with the phase in which spermatogenesis was arrested. Each AZF locus acts at a different phase of spermatogenesis and deletion of each locus causes spermatogenic arrest at a particular stage. On the basis of testicular histology, the deletion of AZFa was associated with the complete absence of germ cells and the presence of Sertoli cells in the seminiferous tubules, characteristic of SCO syndrome, and was associated with azoospermia. The deletion of AZFb was associated with developmental arrest of germ cells at the pachytene stage and led to meiotic maturation arrest. The deletion of AZFc was associated with developmental arrest of germ cells at the spermatid stage, but was also found to be associated with hypospermatogenesis or maturation arrest and was associated with low sperm counts. Thus, deletion of a particular AZF locus results in a characteristic phenotype, and genes at each locus act at a paticular stage of germ cell differentiation.

In the present study patients 1 and 2 had AZFa and AZFb deletions, and testicular cytopathology showed the presence of Sertoli cells and the complete

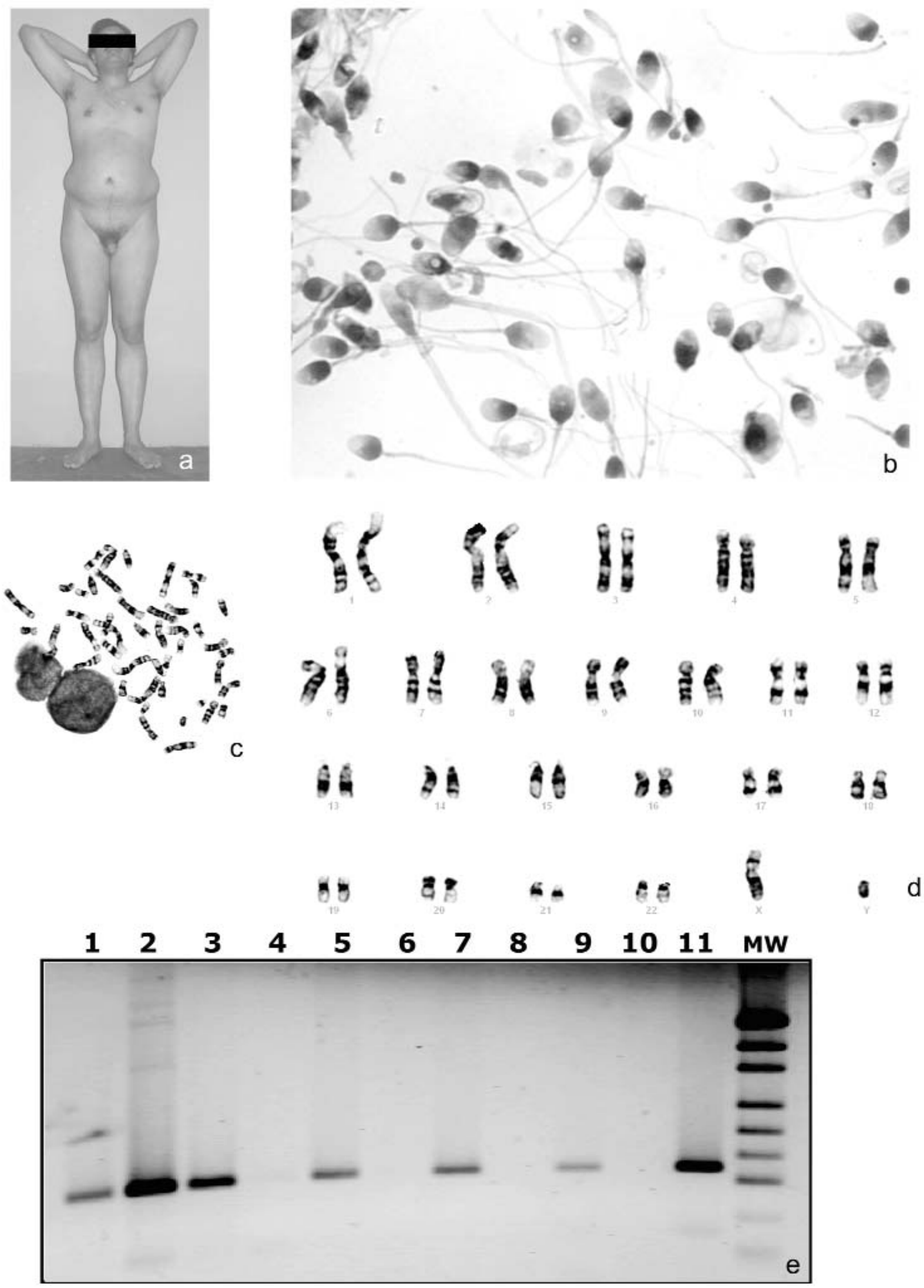

FIGURE 1

A: Phenotype of cytogenetically normal infertile male, infertile with AZFc microdeletion: tall, scant hair in axilla and body, small penis. **B:** Semen analysis photomicrographs showing high percentage of sperm with morphological abnormalities: coiled tail, bent midpiece, thick tail, tapered head, amorphous head (oligoasthenoteratozoospermia). **C** and **D:** Metaphase spread and karyotype showing 46,XY chromosome complement. **E:** Gel photograph showing deletion in the AZFc region (sY255-126 base pairs) in lanes 4, 6,8, and 10.

absence of germ cells (SCO). Patient 3 had AZFa and partial AZFb deletions, and FNAC showed the total absence of germ cells (SCO). Patients 5, 6, 7, and 8 had AZFc deletions. Patients 6 and 8 showed hypospermatogenesis, and in patient 7 there was maturation arrest at the secondary spermatocyte stage. Thus, in patients with AZFc deletion the testicular phenotype may vary.[5,9,10] Dada and associates[10] reported that the heterogenous phenotype observed in AZF deletions may be due to the modifying effect

TABLE I

Phenotypic Features in Men with Yq Microdeletion in the AZF Locus

Patient No.	Azoo/Oligo	Testes Right	Testes Left	FNAC	FSH mIU/mL	STS Deleted	AZF Deletion
1	Azoo	Small	Small	SCO	22	sY84 sY86 sY113 sY116 sY127 sY134	AZFa + AZFb
2	Azoo	Mild hydrocele		SCO	60	sY84 sY86 sY113 sY116 sY127 sY134	AZFa + AZFb
3	Azoo	Small	Varicocele	SCO	31.8	sY84 sY86 sY113 sY116 sY127	AZFa + Partial AZFb
4	Azoo	Cryptorchidism		—	41.3	SY113 SY116 sY127 sY134	AZFb AZFb
5	Azoo	Cryptorchidism		—	6.8	sY254 sY255	AZFc
6	Oligo Sperm count decreased	Small	Small	Hypospermatogenesis	11.8	sY254 sY255	AZFc
7	Azoo	Normal		Maturation arrest (Sec. spermatocyte)	26.2	sY254 sY255	AZFc
8	Azoo	Small	Varicocele	Hypospermatogenesis	44	sY254 sY255	AZFc

AZF, azoospermia factor; FNAC, fine needle aspiration cytology; FSH, follicle stimulating hormone; STS, sequence tagged site,

of one's environment, the expression of various modifying genes, and variable penetrance. Thus several factors—genetic, epigenetic, and environmental—influence the testicular phenotype in men with AZF deletions. Patient 6 showed a decline in sperm count from 1.5 million to 0.2 million over a period of one year. He also had a very high percentage of morphologically abnormal sperm with impaired motility. Similar quantitative decline in semen quality has also been reported in a patient with AZFc deletion.[11] Thus the genotype–phenotype correlation found in the present study is similar to that described in previously published studies.[5] No AZF microdeletions were observed in 50 fertile controls, thus confirming that Yq microdeletions are specific for spermatogenic arrest.

Two cryptorchid patients (patients 4 and 5) showed AZFb and AZFc microdeletions, respectively. Cryptorchidism represents one of the most common congenital anomalies, with a prevalence of 4% to 5% at birth but decreasing by one year to 1%. Cryptorchidism is associated with impaired fertility and increased incidence of testicular cancer.[12] The etiology of testicular descent in the male fetus is multifactorial. Several factors are responsible for normal testicular descent, such as increased intraabdominal pressure, intraabdominal temperature, maternal hormones, normal hypothalamic-pituitary gonadal axis, action of genes (HOX10, INSL-3), and development of gubernaculum. Cryptorchidism, or hidden testis, is associated with spermatogenic impairment resulting from exposure to high intraabdominal temperature. But in men with cryptorchidism with AZF microdeletions, the spermatogenic impairment may be more severe because of the additive effect of high abdominal temperature. Elevation of temperature by 1°C causes depression of spermatogenesis by 14%.[13] The results of these preliminary findings show that AZF microdeletions may be responsible for severe testicular damage. Foresta et al.[13] reported Yq microdeletions in the AZF region in 27.5% cases of cryptorchidism and 25.4% cases with idiopathic infertility. In men with cryptorchidism without Yq microdeletion, surgical management (orchidopexy) at an early age results in improvement in semen quality; but in cryptorchid cases with Yq microdeletion, surgical management does not improve the semen quality. The results of this study show that AZF microdeletions may lead to partial or complete spermatogenic arrest and that the effect of high temperature may further worsen this condition and cause deterioration in semen quality.[9,10,14–16] Hence, awareness of these deletions in cryptorchid cases is critical to the prognosis for these patients and to the management and counseling they are offered.

Varicocele is pathological dilation of the pampiniform plexus of veins and its association with male infertility is well documented. Varicocele occurs far more commonly as an isolated, left-sided lesion (75%–95%). It is associated with a decrease in testicular volume and may also involve the contralateral testis, leading to impaired semen parameters, severe oligozoospermia, and azoospermia.[15,16] The pathophysiology of spermatogenic arrest in varicocele occurs by different mechanisms such as testicular hyperthermia, venous and adrenal reflux,[17] increased venous pressure, alteration of testicular microvasculature,[18,19] defective mitochondrial oxidative phosphorylation or defective energy metabolism,[20] hormonal dysfunction with lower concentration of free testosterone and higher estradiol,[21] autoimmunity,[22] and oxidative damage.[23]

In the present study we had two cases with left-sided varicocele. Both patients 3 and 8 were azoospermic. Patient 3 had AZFa and partial AZFb deletions (STS deleted sY84, sY86, sY127) and patient 8 had AZFc deletion. Testicular phenotype showed SCO in patient 3 and hypospermatogenesis in patient 8. To the best of our knowledge only one or two studies[15] have shown the association of Yq microdeletions in men with varicocele. The prevalence of Yq microdeletion in patients with varicocele and bilateral severe testiculopathy is similar or slightly lower to that found in idiopathic severe testiculopathy.[24] Thus, in these cases, the effect of varicocele may be additive to the spermatogenic impairment caused by AZF deletion. The finding of a genetic etiology in infertile men with varicocele and cryptorchidism suggests that in such patients Yq microdeletion screening should be performed for a proper diagnosis and to avoid unnecessary treatment. The diagnosis of Yq microdeletion in men with varicocele and cryptorchidism is especially important for such patients so that they can be aware of the possibility of transmitting the genetic etiology to their male sons.[10,25]

Patient 6 with AZFc deletion showed a decline in total sperm concentration from 1.6 million to 0.2 million. This decline in semen quantity over a period of time has also been reported by Dada et al.[9,10] Testicular FNAC showed hypospermatogenesis in this patient. As the testicular phenotype deteriorates with time, these men are counseled to undergo sperm cryoconservation for assisted reproduction. Patient 6 was also counseled about the offspring inheriting the same genetic defect. Vogt and Fernandes[26] reported that, in contrast to mouse, bull, and rat spermatogenesis, human spermatogenesis is highly disorganized and that even healthy fertile men may have ejaculate with a high percentage of sperm with morphological abnormality. They also reported that AZFc microdeletions led to a variable phenotype with a significant reduction in sperm count and secondarily to an increased loss of germ cells and progressive decline in semen quality.

All the cases with AZF microdeletion had a normal karyotype, thus proving the importance of PCR to analyze infertility cases. Krausz et al.[8] found that these microdeletions may cause deregulation of gene expression by position effect and interfere with post-transcriptional modification of gene expression, but they postulated that it is equally likely that the deletion results in the absence of genes critical for spermatogenesis. The Y chromosome has the highest spontaneous loss of genetic material in the human genome. This genetic instability arises from the presence of highly repetitive segments—the long and short interspersed repeats—and from a large portion of the Y chromosome (95%) that does not undergo recombination during meiosis. Most of the ancestral genes are functionally intact on the X chromosome, which undergoes crossing over; but because of the lack of X-Y recombination, there is monotonic decline in gene function on the Y chromosome and thus the accumulation of deleterious mutations. The incidence of microdeletions varies from 1% to more than 55% in different studies. These differences in deletion frequency and localization between different studies may be due to the inclusion of varying populations in a study or to the use of different STS primers, or they may reflect genuine population variances such as particular Y chromosome haplotypes, genetic background, or environmental influences. Recent studies have also shown that mutations in the mitochondrial genome result in oligozoospermia, production of sperms with abnormal morphology and impaired motility. In the present study only men with oligozoospermia and azoospermia were considered and the STS primers used were those recommended by the European Academy of Andrology. This setup provides the minimal standardization necessary to determine the variation in deletion frequency in different populations worldwide.

Microdeletions in the AZF region were detected in 4 men with idiopathic infertility, in 2 cryptorchid men, and in 2 men with varicocele. Thus, 6.01% infertile men had microdeletions of the AZF loci. This figure is similar to those reported in Italian, French, and Danish populations, suggesting that the incidence of Y microdeletions is likely to be similar worldwide if a common clinical criteria (patient group) and marker set are used.

Chromosomal and genetic causes of infertility have become very important to identify since the development of assisted reproduction technology, for intracytoplasmic sperm injection now allows men with sperm defects to procreate.[26] These defects can have a genetic origin and thus may be transmitted to the offspring. In order to establish an etiology and provide the most valuable counseling and therapeutics to patients, the genetic etiology of infertility should be established. Diagnostic and therapeutic practices have to keep pace with the rapidly developing insight to the molecular aspects of reproduction in man.

CONCLUSION

Azoospermia factor microdeletions are specific for spermatogenic arrest and lead to oligospermia or azoospermia. Microdeletion analysis using PCR helps determine the frequency and site of gene deletion and thus the testicular phenotype, and also contributes to the determination of an accurate prognosis and ultimately to valuable counseling for couples diagnosed with AZF microdeletions. With the availability of assisted reproduction technology for infertile couples, a genetic etiology is critical for the well being of these prospective parents and their potential offspring.

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